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14. ABSTRACT
The proposed study was aimed at understanding the role of MUC16 cytoplasmic tail (MUC16CT) in MUC16 mediated breast cancer pathogenesis and progression. To achieve this we proposed to understand the role of MUC16CT by carrying out over expression of MUC16CT in BC cells followed by performing various established in vitro and in vivo functional studies. To this end, we have generated FLAG-tagged MUC16CT-114FL and 114dCT stable transfectants in MDAMB231 and MCF7 breast cancer cells. Besides, our cell cycle analysis demonstrated that MUC16CT is responsible for the rapid G2/M transition (hence increased proliferation) compared to vector and 114dCT transfected MDAMB231 cells. Further functional studies are being undertaken. In another aim, we proposed to understand the mechanism of MUC16 mediated breast cancer progression. To this end, we demonstrated that MUC16CT is localized to the nucleus and is enriched on the chromatin bound fraction in a cytoplasmic tail dependent manner. Though the exact functional significance of the finding have not been elucidated, it is anticipated that MUC16CT might be bringing about a global transcriptional change by recruiting to the promoters of various genes probably by interacting with other transcription factors (as MUC16CT as such does not have any DNA-binding domain). In addition, we have demonstrated that MUC16CT is undergoing cleavage in the last SEA domain at a site other than the earlier predicted site. This will help us in accurately defining the role of MUC16CT in breast cancer pathogenesis. In addition, we have also demonstrated that MUC16CT undergoes post-translational modifications such as ubiquitination, which might alter its localization as well as signaling functions and stability. We are in the process of understanding the functional significance of such findings. Besides, mitochondrial localization of MUC16CT is evident. To identify the other potential modifications and/or interacting partners, we are undertaking tandem-affinity purification followed by mass spectrometry to elucidate the molecular mechanisms of MUc16 mediated breast cancer pathogenesis.

15. SUBJECT TERMS: Cytoplasmic tail, nuclear localization, chromatin enrichment, cleavage

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Introduction: Breast cancer (BC) is the major health problem and is the 2nd leading cause of cancer-related deaths in American women, representing 15% of all female cancer deaths (1). Therefore, further efforts toward early diagnosis and therapeutic interventions are needed for the prevention and cure of this malignant disease. Mucins are high molecular weight glycoproteins expressed by epithelial cells lining the luminal surfaces of respiratory and gastrointestinal tracts (2). It has been shown that the deregulated expression of mucins is a prominent characteristic of various types of cancers and inflammatory diseases (2, 3). Particularly, the involvement of membrane bound mucins in various signaling pathways has been a critical determinant and MUC1 mucin is the best characterized with respect to its cytoplasmic tail. MUC16 (also known as CA125) has been used for more than 20 years to characterize the growth and progression of human ovarian cancer (OC), the molecular identity of which was very recently established to be a membrane-bound mucin (MUC16) (4, 5). In addition to OC, an elevated serum and salivary level of CA125 has been detected more in BC patients compared to the healthy controls (6). Besides being used as a diagnostic marker, nothing much was known about MUC16 until its cloning and characterization in 2001 (4, 5). Our recent studies have demonstrated that MUC16 is over expressed in breast cancer and pancreatic cancer patients (7, 8). Based on the sequence, MUC16 is a type-I membrane-bound mucin with a heavily *O*-glycosylated N-terminal domain followed by a C-terminal domain comprising of a tandem repeat region of 156 amino acids repeated over 60 times, several SEA (for Sea-urchin Sperm protein, Enterokinase, and Agrin) modules near the membrane spanning region, a transmembrane region (TM) and a 32-residue cytoplasmic tail (CT) domain (9). Particularly, the cytoplasmic tail domain is interesting due to presence of a stretch of polybasic amino acids RRRKK, which has been shown to be the site of interaction for cytoskeletal proteins like Ezrin/Radixin/Moesin. In addition, our bioinformatics analysis predicted this site to be a potential nuclear localization signal (NLS) and might facilitate its nuclear localization with associated TFs and can modulate the transcriptional regulation of various target genes. Besides, there are several serine, threonine and tyrosine phosphorylation sites facilitating interaction with various intracellular kinase and non-kinase proteins and influence oncogenic signaling pathways. Now we have demonstrated that MUC16 is over expressed in breast cancer patients and induces a rapid G2/M transition by interacting with JAK2 (probably at RRRKK motif of MUC16 and FERM domain of JAK2) in breast cancer cells (7). Based on the above observations I hypothesize that the oncogenic potential of MUC16 associated with BC progression, is in part, mediated by the potential involvement of MUC16CT with diverse oncogenic signaling molecules leading to enhanced mammary tumorigenesis. Two specific aims were laid out for the study.

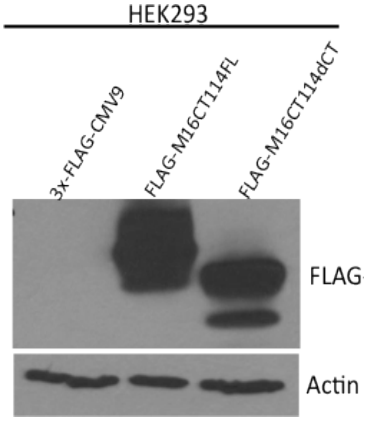

Fig 1. Expression of FLAG-tagged MUC16CT-114FL and dCT constructs in HEK293 cells using p3X-FLAG-CMV9 vector. Immunoblotting was carried out using anti-FLAG antibody. Actin was used as loading control.

Aim 1: *To investigate the MUC16 cytoplasmic tail domain in tumor growth and*

metastasis of BC cells. Aim 2: *To determine the mechanism(s) of MUC16CT mediated BC progression by identifying potential phosphorylation sites and interacting partners of MUC16CT.* To address the first aim, I have already generated stable transfectants of MDAMB231 and MCF7 breast cancer cells with FLAG-tagged MUC16CT114FL and 114dCT, which will be used to carry out all the proposed *in vitro* and *in vivo* functional studies.

Towards Aim 2, I have demonstrated that in fact, MUC16CT is localized to the nucleus and enriched on the chromatin in a cytoplasmic tail dependent manner. Besides, I have shown that it is the last SEA domain where cleavage of MUC16 occurs. MUC16CT undergoes post-translational modifications such as Ubiquitination. Identification of phosphorylation sites, interacting partners and the functional significance of sub cellular localization and post-translational modifications in breast cancer pathogenesis are underway.

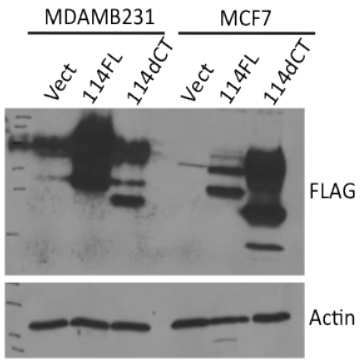

Fig 2. Expression of MUC16CT-114FL and 114dCT in MDAMB231 and MCF7 breast cancer cells. Vector transfected cells were used as control. These cells are stably selected with 400ug/ml of G418.

Body of the Report:

(a) *Generation of MUC16CT constructs with tags either at N-terminus (FLAG) and/or both N- (FLAG) and C-terminus (HA):* Though we had used the EYFP-tagged MUC16CT constructs earlier, where the EYFP tag was present C-terminal to MUC16CT, I generated N-terminal FLAG tagged constructs which are much smaller than the EYFP tag and N-terminal tagging seemed more logical than C-terminal to prevent any sorts of interference. To this end, I cloned the 114 amino acids residue from the C-terminal end with N-terminal FLAG into p3X-FLAG-CMV9 vector. In addition, another construct was made which lacked the C-terminal 32 amino acid long cytoplasmic tail harboring the putative nuclear localization signal and several phosphorylation sites. These constructs were named as MUC16CT-114FL and MUC16CT-114dCT respectively. It has been predicted that MUC16 might undergo cleavage either at the ultimate or penultimate SEA domain from the C-terminus thereby can have two cleavage sites. In order to address this issue I generated three more constructs with FLAG-tag in N-terminus and HA-tag in C-terminus with varying lengths of MUC16CT from the C-terminus (i.e. 200 amino acids harboring the ultimate SEA domain termed as 114-Clvg, 321 amino acids harboring both ultimate and penultimate

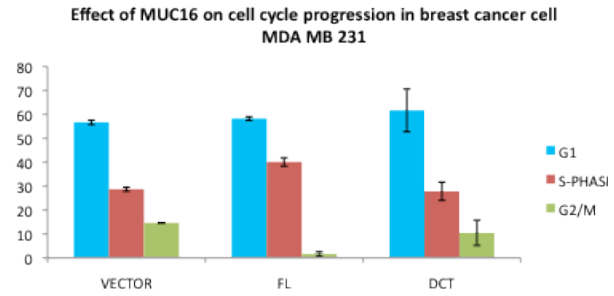

Fig 3. Effect of MUC16CT (FL and dCT) in cell cycle progression in MDAMB231 breast cancer cells. MUC16CT114FL transfected cells have more cells in the S-phase (40%) and less in G2/M phase (1.6%) compared to vector and dCT transfected cells. Similar results were obtained with MUC16 knock down studies in Ref 7.

SEA domains termed as 344-Clvg and exact 114 amino acids termed as FLAG-114FL-HA constructs. Besides, cleavage of MUC16 has been proposed to be carried out by enzymes like MMP7 and neutrophil elastase. Hence, in order to demonstrate which enzyme is responsible for the cleavage, I cloned 114FL-MUC16CT in to pET-32a bacterial expression vector with His-tag on both ends to demonstrate the cleavage *in vitro*. As mentioned in Aim 2, we wanted to identify potential interacting partners for MUC16CT, for which we adopted a tandem affinity purification (TAP) assay, where FLAG-tagged 114FL-MUC16CT was cloned into pZome-1C vector with a TAP-tag (Calmodulin binding protein (CBP)-TEV protease cleavage site-Protein A) in the C-terminus, following which the FLAG-MUC16CT114FL-TAP tag was amplified and cloned into p3X-FLAG-CMV9 vector which has a leader peptide preceding FLAG-tag. Expression of all the constructs was verified by transient transfection into HEK293 cells followed by immunoblotting with either FLAG or HA- antibodies.

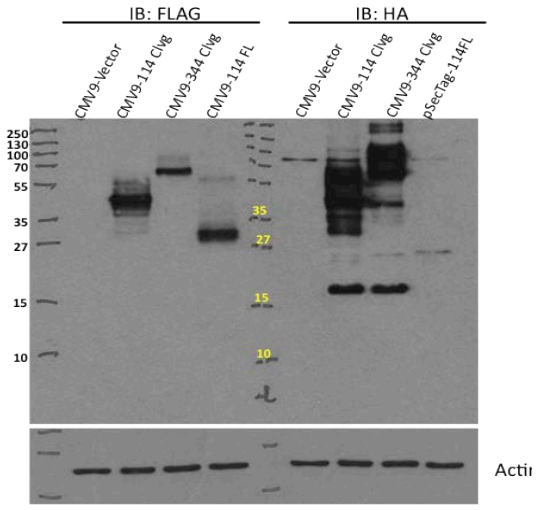

Fig 4. Demonstration of cleavage of MUC16 using constructs with either one SEA (114-Clvg) or two SEA domains (344-Clvg). Immunoblot with C-terminal HA-tagged antibody demonstrates that both the constructs results in the same molecular weight band emphasizing the cleavage in the ultimate SEA domain. Due to post-translational modifications, the apparent molecular weight is higher than the predicted weight, hinting towards a downstream proteolytic cleavage site than the predicted one.

(b) Expression of FLAG-tagged MUC16CT-114FL and 114dCT constructs: After generating the FLAG-tagged 114FL and CT deleted constructs, transient transfections of HEK293 cells were carried out and p3X-FLAG-CMV9 vector was used as control. Expression of the full length and CT deleted proteins were detected by immunoblotting using anti-FLAG antibody. As shown in **Fig 1**, the observed molecular weights for both the FLAG-tagged MUC16CT-114FL (28kDa as opposed to predicted 18kDa) and 114dCT (24kDa as opposed to predicted 13kDa) were higher than the sequence predicted weight, suggesting probable post-translational modification of MUC16 cytoplasmic tail. To this end, we have shown that MUC16 cytoplasmic tail undergoes ubiquitination (please see section ***f***), which is responsible for the increased molecular weight. As there are potential lysine and cysteine residues, which could get ubiquitinated, I am currently carrying out mutagenesis study to examine which residues are critical for ubiquitination. This could have interesting functional consequences, which is under investigation.

(c) Generation of stable transfectants of MDAMB231 and MCF7 breast cancer cells with MUC16CT-114FL and 114dCT constructs: To understand the role of MUC16 cytoplasmic tail (32 amino acid intracellular portion), the above mentioned constructs were transfected into MDAMB231 and MCF7 breast cancer cells and were stably selected using 400ug/ml of G418 and p3X-FLAG-CMV9 vector transfected cells are used as control. Expression of FLAG-tagged MUC16CT-114FL and 114dCT constructs were confirmed by immunoblotting with anti-FLAG antibody (**Fig 2**). Our preliminary

studies with the MDAMB231 transfected cells (vector, FL and dCT) demonstrates that 114FL transfected cells have higher percentage of cells in the S-phase (40.1%) and less in G2/M phase (1.6%) compared to vector and dCT transfected cells (vector: S-phase = 28.6% and G2/M =14.4%; dCT: S-phase = 27.8% and G2/M = 10.4%) (**Fig 3**). This validates our recently published study that down regulation of MUC16 in MDAMB231 cells prevents the G2/M transition leading to accumulation of cells in the S-phase, which was shown to be mediated by its interaction with JAK2. This function of MUC16 may be mediated by the cytoplasmic tail probably by mediating its interaction through the RRRKK polybasic motif, but needs further experimental validation. Further functional characterizations of the transfectants (such as growth kinetics, motility, invasion, cell cycle and apoptosis assays) are underway.

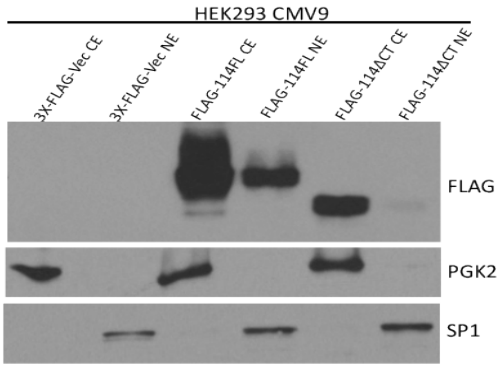

Fig 5. Demonstration of subcellular localization of MUC16CT by cell fractionation method. HEK293 cells were transiently transfected with 3X-FLAG vector, 3X-FLAG114FL, 3X-FLAG114ΔCT followed by cytoplasmic and nuclear extraction. As observed in immunofluorescence, deletion of cytoplasmic domain abrogates the localization of MUC16CT into nucleus. PGK2 is used as a marker for cytoplasmic and SP1 as a marker for nuclear lysates to demonstrate the purity of fractionation.

(d) *<u>Cleavage of MUC16 is at the ultimate SEA domain and down stream to the predicted cleavage site:</u>* To demonstrate the cleavage of MUC16, I generated the 114-Clvg and 344-Clvg constructs with N-ter FLAG and C-ter HA epitope tagged constructs (described in section ***a***). These constructs were transiently transfected into HEK293 cells and were probed with both FLAG and HA-tagged antibody (**Fig 4**). This resulted in 17-18kDa molecular weight HA-tagged MUC16CT (which may have undergone ubiquitination as mentioned before) in both cases, demonstrating a downstream cleavage site than the predicted. This suggests, probably the unmodified molecular weight of MUC16CT is approximately 7-8kDa (with single ubiquitination gives 17-18kDa band). In that case the cleavage is probably occurring downstream to the previously predicted 114 amino acids from the C-ter end. Further mutational studies are under way to confirm the findings. To demonstrate whether MMP7 and/or neutrophil elastase is responsible for

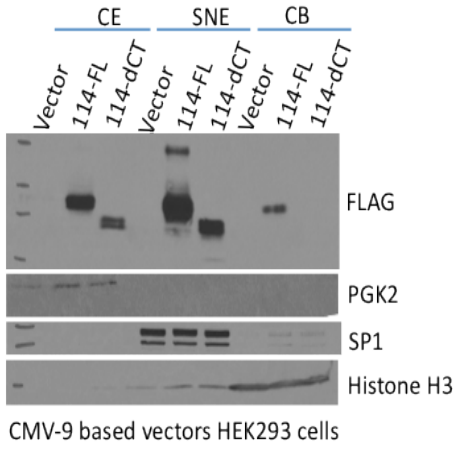

Fig 6. Demonstration of chromatin enrichment of MUC16CT in a tail dependent manner. Transiently transfected HEK293 cells were subjected to sub cellular fractionation, where the nuclear fractions were separated in to soluble nuclear (SNE) and chromatin bound (CB) nuclear fractions. CE stands for cytoplasmic extracts. Only MUC16CT114FL is bound to chromatin, not the dCT. PGK2, SP1 and Histone H3 were used as loading and purity control for CE, SNE and CB fractions respectively.

cleavage of MUC16, I have cloned and expressed His-tagged MUC16CT-114FL in pET32-a bacterial expression vector. Currently, I am standardizing the purification protocol as most of the proteins are found in the insoluble fractions.

(e) *Nuclear localization of MUC16CT is intracellular cytoplasmic tail dependent:* Using the above-mentioned constructs with and without intracellular cytoplasmic tail domain of MUC16CT in HEK293 cells, I have demonstrated that MUC16CT114FL localizes to nucleus whereas MUC16CT114dCT fail to do so (**Fig 5**). As the intracellular cytoplasmic tail domain harbors RRRKKE motif, we think this to be a potential NLS as has been predicted by bioinformatics analysis. Further, to demonstrate that it is the true NLS, I am carrying out cassette mutagenesis of the NLS to alanine residues to demonstrate the same. In addition to nuclear localization, I have also demonstrated chromatin enrichment of MUC16CT in a cytoplasmic tail dependent manner (**Fig 6**). This implies that, MUC16CT is probably localized into the nucleus using the NLS and is bound to the transcription factors thereby enriching on the chromatin (MUC16CT does not have any obvious DNA-binding domain) and can influence the transcriptional events leading to enhanced tumorigenesis. To this end, I will be performing Chromatin Immunoprecipitation – sequencing (ChIP-Seq) analysis to understand the promoter enrichment of MUC16CT affecting global transcriptional changes.

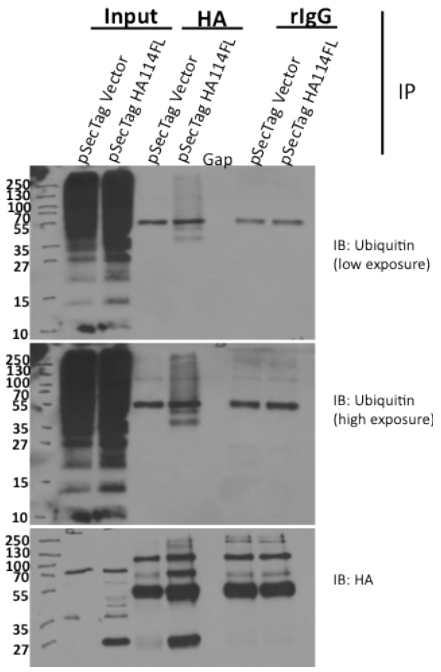

Fig 7. Ubiquitination of MUC16CT. N-terminal HA-tagged MUC16CT-114FL constructs were transiently transfected into HEK293 cells, followed by immunoprecipitation of MUC16CT using anti-HA antibody. Following immunoprecipitation, the lysates were immunoblotted with HA (to demonstrate efficient pull down of MUC16CT) and Ubiquitin antibody. Immunoblot with Ub-antibody demonstrates that MUC16CT-114FL is ubiquitinated compared to vector transfected control cells. Rabbit IgG was used to demonstrate specificity of HA-Ab.

(f) *Post-translational modifications of MUC16CT*: As mentioned earlier, we observed higher molecular weight bands for both MUC16CT-114FL and MUC16CT-114dCT proteins than the sequence dictated molecular weight. So we investigated whether MUC16CT undergoes any sorts of post-translational modifications. As the molecular weight differences were observed in a multiple of 10kDa, the logical choice was either Ubiquitination or Sumoylation. By carrying out immunoprecipitation studies, I have demonstrated that MUC16CT undergoes Ubiquitination (**Fig 7**). Sumoylation was not detected at this point. It appears that both lysine and cysteine residues undergo ubiquitination, as we see increased molecular weight of MUC16CT-114dCT which is devoid of lysine residues. Further mutagenesis studies are underway to determine the critical residues important in the ubiquitination process. Ubiquitination of MUC16CT can have significant impact in the functions of MUC16CT such as localization, signaling functions and interactions with other proteins.

Besides the above-mentioned findings, our preliminary study suggests

mitochondrial localization of MUC16CT (**Fig 8**), the significance and mechanism of which will be addressed in future studies. In addition, to identify the interacting partners of MUC16CT, I have cloned and expressed the FLAG-tagged MUC16CT114FL-CBP-Protein-A cassette into p3X-FLAG-CMV9 vector and the expression was verified using anti-FLAG antibody (**Fig 9**). Tandem affinity purification followed by mass spectrometry will be performed to identify potential modifications (such as Ubiquitination, Sumoylation, Phosphorylation etc), and interacting partners. In addition, the effect of sub cellular localization, modifications etc will be addressed to understand the potential implications of MUC16CT in mammary tumorigenesis.

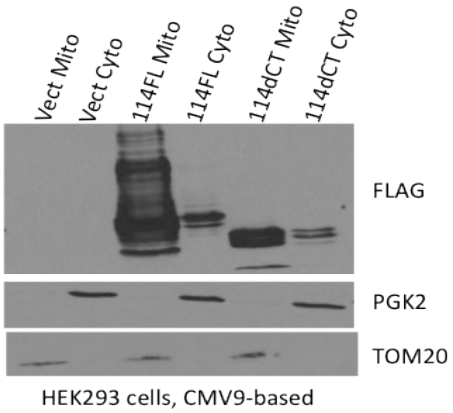

Fig 8. Mitochondrial localization of MUC16CT. HEK293 cells were transiently transfected with MUC16CT114FL and 114dCT constructs, followed by density gradient separation of mitochondria and immunoblot analysis using anti-FLAG antibody. PGK2 and TOM20 antibodies were used to demonstrate the purity of cytoplasmic and mitochondrial fractions respectively.

Key Research Accomplishments:

- Cloning and expression of different lengths of MUC16CT using p3X-FLAG-CMV9 vector.
- Generation of stable transfectants of MDAMB231 and MCF7 with MUC16CT constructs.
- Cleavage of MUC16 in the ultimate SEA domain.
- Nuclear localization and chromatin enrichment of MUC16CT is intracellular cytoplasmic tail dependent.
- Ubiquitination of MUC16CT.
- Mitochondrial localization of MUC16CT.
- Cloning and expression of MUC16CT-114FL into pET-32a bacterial expression vector.
- Cloning and expression of FLAG-tagged MUC16CT-114FL into pZome-1C (TAP vector) vector followed by cloning into p3X-FLAG-CMV9 vector.

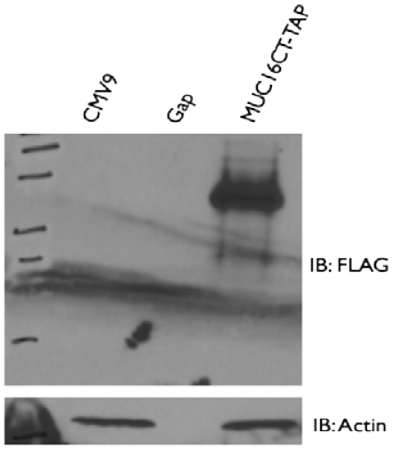

Fig 9. Cloning and expression of FLAG-tagged MUC16CT114FL along with the TAP cassette (CBP-TEV-Protein A) into p3X-FLAG-CMV9 vector. This will be subsequently used for tandem affinity purification followed by mass spectrometry to identify potential interacting partners and/or modifications.

Reportable Outcomes:

Besides, above-mentioned key accomplishments, I have been involved in studies related to full length MUC16 in breast and other cancers , which have resulted in two research publications outlined below.

1) Lakshmanan I, Ponnusamy MP, **Das S,** Chakraborty S, Haridas D, Mukhopadhyay P, Lele SM, Batra SK. (**2012**) MUC16 induced rapid G2/M transition via interactions with JAK2 for increased proliferation and anti-apoptosis in breast cancer cells. **Oncogene** 16; 31 (7): 805-17.
2) Haridas D, Chakraborty S, Ponnusamy MP, Lakshmanan I, Rachagani S, Cruz E, Kumar S, **Das S**, Lele SM, Anderson JM, Wittel UA, Hollingsworth MA, Batra SK. (**2011**) Pathobiological implications of MUC16 expression in pancreatic cancer. **PLoS One** 6(10).

Conclusions and future directions: The above-mentioned studies demonstrate that MUC16 plays a critical role in breast cancer pathogenesis and as we have hypothesized MUC16CT plays an important role in this process. This is partially evident where MUC16 mediated rapid G2/M transition of BC cells (Lakshmanan et al.,) was demonstrated to be mediated by MUC16CT114FL transfected MDAMB231, but not by vector or MUC16CT114dCT transfected cells. Further functional characterizations are underway. To identify the mechanism of MUC16CT mediated tumorigenesis, we have demonstrated that MUC16CT is localized in to the nucleus and is enriched on the chromatin in cytoplasmic tail dependent manner. Functional implications of such findings will be addressed by carrying out genome wide ChIP-Seq studies. Besides, we have observed mitochondria localization of MUC16CT, post-translational modifications such as ubiquitination etc., functional significance of which is under investigation. To identify further modifications and/or interacting partners, I am carrying out tandem-affinity purification followed by mass spectrometry. In future, I will be carrying out several mutagenesis studies to demonstrate the specificity of cleavage, ubiquitination, phosphorylation etc. of MUC16CT and its relevance in mammary tumorigenesis.

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